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RESEARCH PAPER

Factors affecting the accumulation of 9-methoxycanthin-6-one in callus cultures of *Eurycoma longifolia*

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Abstract: A study was conducted to improve 9-methoxycanthin-6-one productivity (potential anti-tumour compound) from callus cultures of *Eurycoma longifolia* (Tongkat Ali). Several factors affecting 9-methoxycanthin-6-one production in callus cultures such as different medium compositions and physical factors were investigated and analyzed. Results show that a higher production of 9-methoxycanthin-6-one (3.84 mg·g^{-1} DW (Dry Weight)) is obtained from callus cultured in ¼ MS basal media. At fructose of 2% (w/v), the production of 9-methoxycanthin-6-one (4.59 mg·g^{-1} DW) is promoted to gain the highest yield, compared to other carbon sources tested. The addition of 2.0-mg·L^{-1} dicamba also increases 9-methoxycanthin-6-one production (12.3 mg·g^{-1} DW). Higher production of 9-methoxycanthin-6-one was obtained at pH 5.5 (1.53 mg·g^{-1} DW). Production of 9-methoxycanthin-6-one (2.34 mg·g^{-1} DW) in callus cultures is also increased when the medium is added with 1×10^{-1} μM phenylalanine. This study suggests that the successful production of 9-methoxycanthin-6-one *in vitro* cultures has a potential in large-scale production using bioreactor technology.

Keywords: *Eurycoma longifolia*; callus culture; 9-methoxycanthin-6-one; dry weight

Introduction

Eurycoma longifolia Jack is commonly used in local traditional medicine against a variety of diseases (Chan et al. 1991). This plant is popularly sought as a single ingredient or as a mixture with other herbs in herbal remedies. The roots of *E. longifolia* are used in traditional medicine as a cure for persistent fevers and malaria (Ang et al. 1995) and in treatment for dysentery, glandular swelling, and tertian malaria (Kordono et al., 1991). Potential medicinal value from this plant is also reported to increase male virility (Kuo et al. 2003).

Medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries depend, in part, on plants for the production of pharmaceutical compounds (Chand et al. 1997). Alkaloids are important secondary plant products and are used for useful drugs. An important alkaloid, 9-methoxycanthin-6-one was significant cytotoxicity against human lung cancer (A-549) and human breast cancer (MCF-7) cell lines (Kuo et al. 2003). Meanwhile, Nurhanan (2002) reported possibly an anti-proliferative effect in ovarian cancer cells. Kardono et al. (1991) reported that 9-methoxycanthin-6-one can be obtained from the roots of *E. longifolia*. This compound is also found to produce in callus cultures of *E. longifolia*. The objective of the present study was to investigate effects of various factors affected by manipulating medium composition on the accumulation of 9-methoxycanthin-6-one in callus cultures of *E. longifolia* (Tongkat Ali).

Materials and methods

Sterilization of explants

E. longifolia plant was grown at the Laboratory of Natural Product Discovery, University Putra Malaysia (UPM), Serdang, Selangor. The explants collected from *E. longifolia* were washed under running of the tap water for 30 min. Two drops of Tween-20 (Sigma) were added as wetting agent into 15% commercial Clorox solution and the explants were soaked in the sterile solution for 30 min. The explants were then rinsed for 5, 10, and 20 min with 100 mL of sterile distilled water. The sterilized explants were cut into small pieces and then transferred into vials (8.4 cm × 2.4 cm).

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Multiplication and maintenance

Callus induced from the explants (leaf, petiole, rachis, stem, tap root, fibrous root, cotyledon and embryo), which formed at the edge of the explants, was excised and transferred to fresh medium. Callus cultures were multiplied and maintained by subculturing onto the MS medium (2.0 $mg \cdot L^{-1}$of 2,4-D) at an interval of three weeks. Calluses obtained from three weeks old callus cultures were used for treatments.

Effect of different medium composition on callus growth and 9-methoxycanthin-6-one production

Basal media: Four basal media tested are MS (Murashige et al. 1962), SH (Schenk et al. 1972), WH (White 1963) and B5 (Gamborg et al. 1968). All the culture media were supplemented with 3% (w/v) sucrose. Iron was supplied as Fe NaEDTA. Quarter (¼×), half (½×), full (1×) and double strength (2×) of these different basal media nutrient were investigated. Only the macronutrient salts of these basal media were varied while other nutrient components were maintained.

Carbon sources: The effects of different carbon sources such as sucrose, glucose, fructose, sorbitol, and mannitol on growth and 9-methoxycanthin-6-one production of callus cultures were individually investigated. The callus cultures are derived from leaf, petiole, stem, rachis, tap root, fibrous root, cotyledon and embryo in full strength of MS medium supplemented with 2.0 $mg \cdot L^{-1}$ of 2,4-D. The concentration of each carbon source ranged from 0 – 5 % (w/v).

Plant growth regulators (PGRs): Five types of PGRs tested, 2, 4-Dichlorophenoxyacetic acid (2,4-D), 3,6-Dichloro-o-aniscic acid (dicamba), 4-Amino-3,5,6-trichloropicolinic acid (picloram), α-Naphthaleneacetic acid (NAA), and Indole-3-acetic acid (IAA) were used to induce the callus from leaf, petiole, rachis, stem, tap root, fibrous root, cotyledon and embryo. The effects of exogenously supplied plant growth regulators (PGRs) on callus growth and 9-methoxycanthin-6-one production were determined at concentrations range between 0 and 5.0 $mg.L^{-1}$in full strength MS medium supplemented with 3 % (w/v) sucrose. The PGRs were added into the basal salt solution. All PGRs were purchased from Sigma (USA). The callus growth and 9-methoxycanthin-6-one production were then investigated.

pH of medium: The initial pH values were adjusted at 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 using either NaOH of 1N or HCl of 1N at room temperature. MS medium was used as the basal medium for this study supplemented with 3% sucrose. Each treatment was conducted in 10 replicates. Treated explants were harvested starting from the first week to seven weeks to analyze the pH of the medium, growth performances, and 9-methoxycanthin-6-one production.

Amino acids: Three types of amino acids used, DL-tryptophan (MW: 204.2), phenylalanine (MW: 165.2), and L-tryrosine (MW: 181.2), were purchased from Sigma (USA). Stock solutions of amino acids were individually prepared by diluting it in distilled water, and further filter sterilized with a 0.2 µM-polyethersulfone membrane (Whatman). A series of concentrations (0, 1×10^{-4}, 1×10^{-3}, 1×10^{-2}, 1×10^{-1}, 1.0, 1×10^{1}, 1×10^{2}, 1×10^{3}, 1×10^{4}µM) were then prepared from stock solution and added into steriled 10 mL of MS medium supplemented with 3 % (w/v) sucrose.

Extraction of 9-methoxycanthin-6-one

The extraction procedures for 9-methoxycanthin-6-one were modified from Choo and Chan (2002). The dried samples of one gram were ground in pestle and mortar, then mixed with 20-ml solvent (CH_3OH : $CHCl_3$ at 4:1) and incubated in ultra sonic bath for 15 min. HPLC analyses of the extracts were carried out. Standard for 9-methoxycanthin-6-one compound was kindly given by Professor Chan Kit Lam, School of Pharmaceutical and Universiti Sains Malaysia (USM).

Analysis of 9-methoxycanthin-6-one using HPLC

The crude extract of Tongkat Ali was analyzed with an Agilent 1100 Series HPLC (USA) comprising Agilent Chem Station for LC System, a manual injector with external 20µL-sample loop, a diode array detector, a quaternary pump and vacuum degasser. Reversed-phase separations were conducted using a 3.9-mm ×150-mm I.D. Nova Pak C18 60A steel cartridge column; fitted at 4 µm (Waters Associates, USA, Part no. 36975) containing dimethyloctadecylsilyl boundedamorphous silica and methanol (CAS no. 67-56) was used at a flow rate of 1.0 $mL \cdot min^{-1}$ at room temperature. The mobile phase consisted of a mixture of acetonitrile and distilled-deionised water (Elga, England) (pH 2.5) acidified with trifluoroacetic acid (TFA) (Sigma, USA) to pH 2.5 (40:60). The total running time is 30 min. The detection of 9-methoxycanthin-6-one compounds was monitored at 272 nm. The reference of 9-methoxycanthin-6-one was dissolved in methanol (HPLC grade). The concentration of identified compounds was determined by external standard method.

Results and discussion

Effect of different media compositions on callus growth and (9-methoxycanthin-6-one) content

Callus culture in full strength of MS basal medium supplemented with 3% (w/v) of sucrose produced the highest biomass (0.08 g DW), (Fig. 1). After 5 weeks of culture, the growth performance of callus in MS basal media is significantly different, compared to the B5, SH and WH medium. The MS basal medium also has highest 9-methoxycanthin-6-one content (3.84 $mg.g^{-1}$ DW) followed by B5 (1.54 $mg \cdot g^{-1}$ DW), SH (1.46 $mg \cdot g^{-1}$ DW), and WH (0.92 $mg \cdot g^{-1}$ DW). Callus culture was used for the quantification of 9-methoxycanthin-6-one compound using HPLC (Fig. 1 (b)). This result is similar to the production of anthocyanin in callus cultures of *Daucus carota* (Narayan et al. 2005).

Effect of different MS nutrient compositions on growth and 9-methoxycanthin- 6-one content

Results of different strength of MS medium were shown in Fig. 2 (a). Different MS nutrient strengths were used for supporting cell

growth. The highest biomass dry weight production of 0.1 g was obtained in full MS strength medium, followed by double, half and quarter MS strength medium. Referring to Fig. 2 (b), the highest 9-methoxycanthin-6-one content was obtained in (¼ MS) medium strength (4.97 mg·g^{-1} DW). Meanwhile, lower 9-methoxycanthin-6-one content was obtained in double strength MS (0.7 mg·g^{-1} DW) medium. The results agreed with that of solasodine production of *Solanum mauritianum* (Drewes et al. 1995a) callus cultures. The nutrient concentration of a particular basal medium was also previously reported as the greatest contribution towards the variation of solasodine production in callus cultures of *Solanum aviculare* (Kittipongpatana et al. 1998). According to Rhodes et al. (1990) and Drewes et al. (1995a), the capability of different basal media formulation in supporting plant cell growth and the synthesis of plant secondary metabolites is linked to the ionic balance in the medium. Drewes et al. (1995b); Lipavska and Vreugdenhil (1996) stated that it is important to find a suitable nutrient concentration of basal medium because lower concentration of nutrient components is not enough to support cells growth. Higher concentration of nutrients may become toxic and cause an osmotic stress for plant cell cultures.

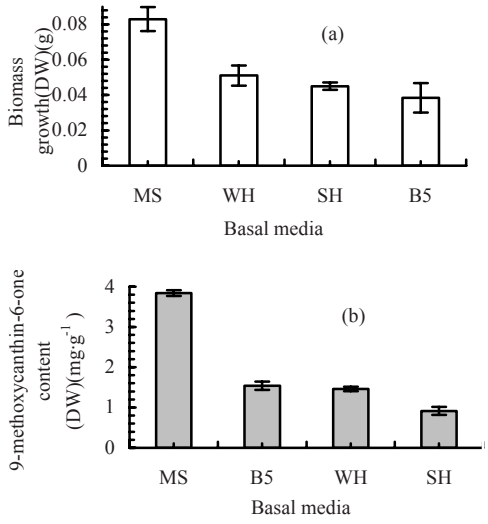

Fig. 1 Callus growth and 9-methoxycanthin-6-one productions of *Eurycoma longifolia* callus cultures in different basal media. (a) biomass growth, (b) 9-methoxycanthin-6-one content. Bar indicates that the standard deviation of biomass growth is from seven replicates and 9-methoxycanthin-

The effects of different phytohormones on growth and 9-methoxycanthin-6-one production are shown in Fig. 3 (a). From the five different auxins tested for callus growth performance, 2,4-D, picloram and dicamba produced the highest dry weight per culture (Fig. 3 (b)). The total dry weight at 0.08 g was obtained for 2,4-D and 0.09 g DW for both picloram and dicamba, respectively. However, the PGRs have no obvious effect on 9-methoxycanthin-6-one production. Surprisingly, the content of 9-methoxycanthin-6-one in per gram in dry weight tissue is higher at 2.0 mg·L^{-1} dicamba (12.3 mg·g^{-1} DW), followed by 2.0, 3.0 and 4.0 mg·L^{-1}NAA (10.7, 9.63 and 8.9 mg·g^{-1} DW) and 2.0 mg·L^{-1} IAA (9.2 mg·g^{-1} DW), respectively. Meanwhile, the supplementation of 2,4-D and picloram in culture medium resulted in very poor yield of 9-methoxycanthin-6-one. A lower biomass dry weight is produced by NAA and IAA. They may not be suitable as phytohormones for *Eurycoma longifolia* callus cultures. In contrast, Fernandes-Ferreira et al. (1992) reported that the NAA and IAA stimulated biosynthesis of triterpenoids, steroid, and sterols in other species of plant tissue cultures.

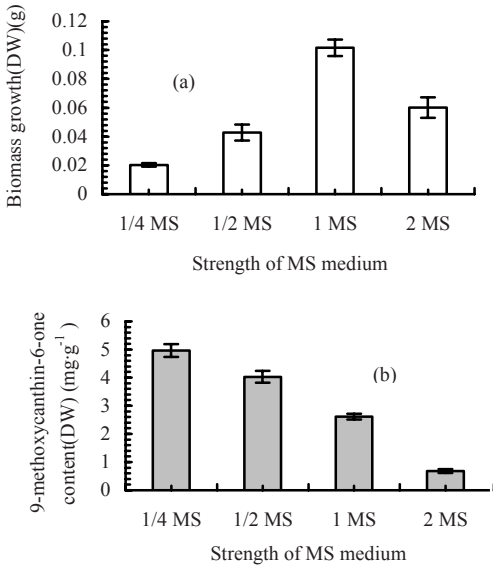

Fig. 2 Callus growth and 9-methoxycanthin-6-one production of *Eurycoma longifolia* callus cultures in different MS nutrient strengths. (a) biomass growth, (b) 9-methoxycanthin-6-one content. Bar indicates that the standard deviations of biomass growth are from seven replicates and 9-methoxycanthin-6-one is from three replicates.

Effects of different carbon sources on growth and 9-methoxycanthin-6-one content

The growth of callus cultured in MS basal medium that is supplemented separately with sucrose, fructose, glucose, sorbitol and mannitol at concentrations of 1%–5% (w/v respectively) was examined. The callus has the best growth when it is cultured in basal medium supplemented with sucrose of 3% (w/v) (Fig. 4 (a)) and reaches maximum (0.09 g) at sucrose of 3% (w/v). The addition of excessive sucrose (above 4% (w/v)) restricts callus growth. Meanwhile, the callus growth performance in the supplementation of 2% (w/v) fructose and 3% glucose is similar to that of 3% (w/v) sucrose. The biomass dry weight production in treatment containing 2% (w/v) glucose is 0.06 g. The highest biomass dry weight in 2% (w/v) fructose treatment is 0.06 g. However, the culture medium with sorbitol and mannitol has very poor callus growth. The effects of different carbon sources on 9-methoxycanthin-6-one production are shown in Fig. 4 (b). Fructose at 2%–4% (w/v) promotes the highest yield of 9-methoxycanthin-6-one and reached maximum yield at 2% (w/v) (9-methoxycanthin-6-one content is 4.59 mg·g^{-1}DW). Surprisingly, the content of 9-methoxycanthin-6-one in a basic per gram dry weight tissue is lower at higher concentration of sucrose.

Meanwhile, the content of 9-methoxycanthin-6-one in a basic per gram dry weight tissue is higher at lower concentration of mannitol at 3% (w/v) and 2.24-mg·g^{-1} DW tissues.

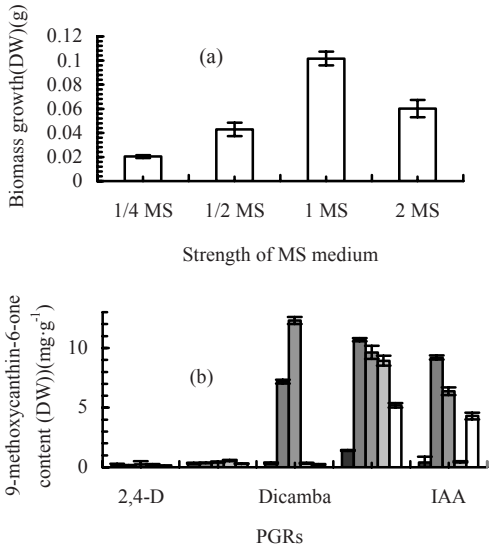

Fig. 3 Effect of different phytohormones concentrations on callus growth and 9-methoxycanthin-6-one production of *Eurycoma longifolia* callus cultures. (a) biomass growth, (b) 9-methoxycanthin-6-one content. Bar indicates that the standard deviations of biomass growth are from seven replicates and 9-methoxycanthin-6-one is from three replicates.

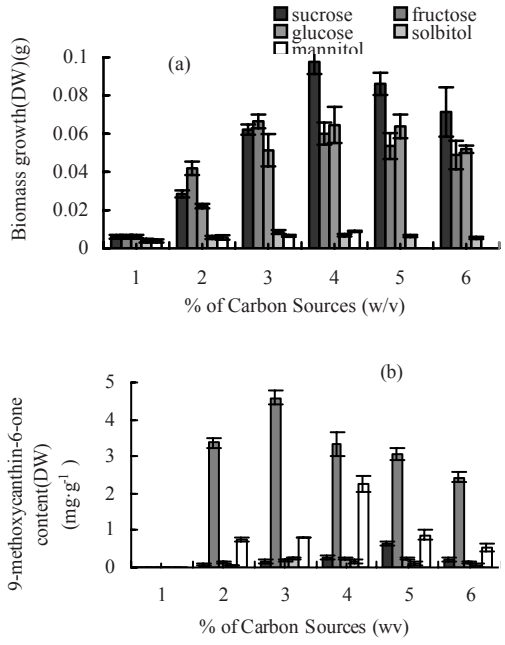

Fig. 4 Callus growth and 9-methoxycanthin-6-one production of *Eurycoma longifolia* callus cultures in different carbon sources. (a) biomass growth, (b) 9-methoxycanthin-6-one content. Bar indicates that the standard deviations of biomass growth are from seven replicates and 9-methoxycanthin-6-one is from three replicates.

Effects of different pH on callus growth and 9-methoxycanthin-6-one content

The effect of different pH on callus growth and 9-methoxycanthin-6-one production was observed at week 5 (Fig. 5(a)). Decrease or increase in the pH of the usual MS basal medium does not improve the callus growth performance significantly. The highest biomass dry weight is obtained at pH 5.5 (0.09 g DW, respectively). The highest 9-methoxycanthin-6-one production is recorded at pH 5.5 (1.53 mg·g^{-1} DW, respectively) (Fig. 5 (b)). This result is similar to the production of swainsonine (159 μg) in transformed root cultures of *Swainsona galegifolia* at pH 5.7 (Ermayanti et al. 1994).

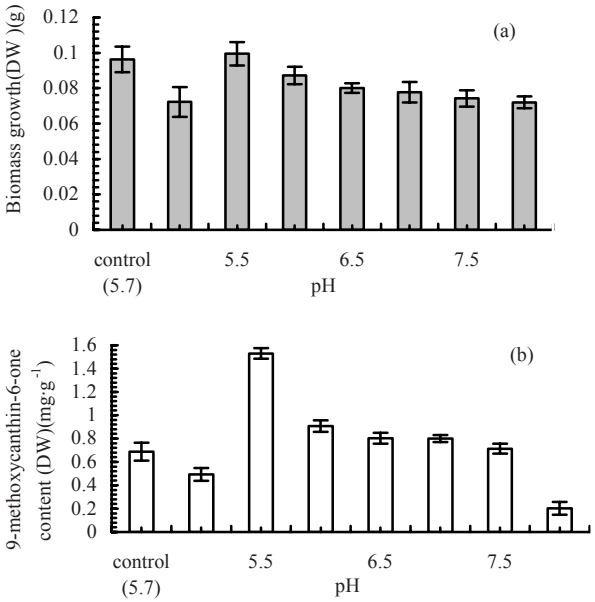

Fig. 5 Changing pH profile in medium culture with respect to callus growth and 9-methoxycanthin-6-one production of *Eurycoma longifolia* callus cultures. (a) biomass growth, (b) 9-methoxycanthin-6-one content. Bar indicates that the standard deviations of biomass growth are from seven replicates and 9-methoxycanthin-6-one is from three replicates

Effects of different amino acids on callus **growth and 9-methoxycanthin-6-one content**

According to Robinson (1981), tryptophan, phenylalanine and tyrosine has been applied into the culture medium as precursors in order to enhance indole alkaloid production. The same method was applied to enhance 9-methoxycanthin-6-one in this study. The tryptophan, phenylalanine and tyrosine at a series of concentrations (0-1×10^4 μM) were aseptically added into sterile MS medium solution. The results Fig. 6 (a) show that the addition of tryptophan, phenylalanine and tyrosine does not affect the callus growth performance of callus cultures of *E. longifolia* significantly at the concentrations from 0 to 1×10^4 μM. A dramatic reduction of biomass growth of callus is only observed at higher concentration. Meanwhile, 9-methoxycanthin-6-one contents are significantly enhanced up to 2.34, 1.83 and 1.45 mg·g^{-1} DW at 1×10^{-1} μM of phenylalanine, 1×10^2 μM of tryptophan, and 1×10^1 μM of tyrosine, respectively, which are 1.84–3.84 folds higher than that of the control (Fig. 6 (b)). These results are also similar to the accumulation of anthocyanins in *Vitis vinifera* cells stimulated by phenylalanine (Krisa et al. 1999). They reported that the

anthocyanin content is 1.2 times greater in the presence of 1×10^{-1} μM phenylalanine. Meanwhile, the addition of 1-mM phenylalanine to the culture medium significantly increased the production of taxol from callus cultures of *Taxus baccata* (Cusido et al. 1999).

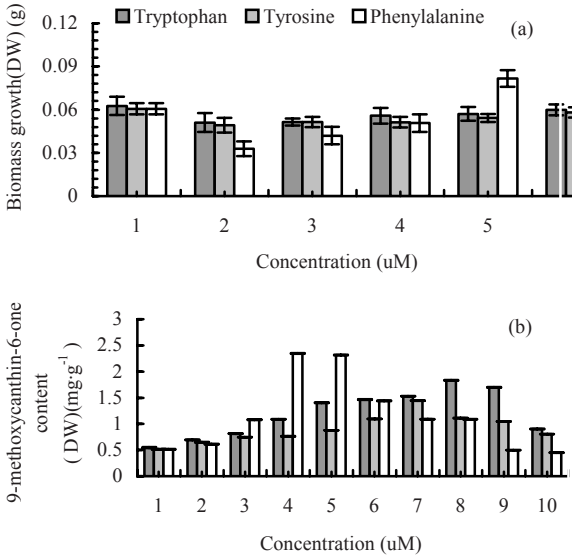

Fig. 6 Callus growth and 9-methoxycanthin-6-one production of *Eurycoma longifolia* callus cultures by addition of phenilalanine, tryptophan and tyrosine. (a) biomass growth, (b) 9-methoxycanthin-6-one content. Bar indicates that the standard deviations of biomass growth are from seven replicates and 9-methoxycanthin-6-one is from three replicates.

Conclusions

The present study shows that a higher production of 9-methoxycanthin-6-one in callus cultures could be obtained by using ¼ MS basal medium (9-methoxycanthin-6-one content was 4.97 $mg\cdot g^{-1}$ DW) supplemented with 2% (w/v) fructose. The addition of 2.0 $mg\cdot L^{-1}$ dicamba can also increase the 9-methoxycanthin-6-one production. Callus cultures are capable to grow in pH range of 5.5 to 6.0. Greater amount of 9-methoxycanthin-6-one is obtained at pH 5.5. Production of 9-methoxycanthin-6-one in callus cultures is also increased when cultures are supplemented with phenylalanine of 1×10^{-1} μM (9-methoxycanthin-6-one content was 2.34 $mg\cdot g^{-1}$ DW). The results indicate that the combination of different basal media, carbon sources, phytohormone, pH and amino acids could be a useful tool in developing rational strategies to enhance the production of 9-methoxycanthin-6-one *in vitro*. Successful production of 9-methoxycanthin *in vitro* cultures can be a potential system in a large-scale production using bioreactor technology.

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